ISBIO2014

GENERAL INFORMATION
AND
BOOK OF ABSTRACTS

Training Course
UCIBIO@REQUIMTE-FCT-UNL

October 6-12, 2014
http://eventos.fct.unl.pt/isbio2014

Integrative Structural Biology tools for the study of protein-ligand interactions

INSTRUCT-supported training course, co-organized by COST action 1306 - Understanding Movement and Mechanism in Molecular Machines

COURSE OBJECTIVES

The study of protein-ligand interactions is fundamental to the understanding of biological systems and is at the heart of the drug discovery process.

There are many biophysical methods available to study protein-ligand interactions that span from calorimetric techniques to spectroscopic and structural methods. Young researchers are usually familiar with one or more of these methods but often do not have the opportunity to address the problem from an integrated structural biology perspective.

In this course our main objective is to illustrate the added value of a structural biology approach to the study of protein-ligand interactions by combining four core methodologies, **X-ray Crystallography**, **NMR**, **Carbohydrate Microarrays** and **ITC** from a hands-on perspective, giving emphasis to their limitations and complementarities.

To achieve this, and to better illustrate the integrative context, the students will receive training by working in a study-case, a protein that recognizes and binds to carbohydrates, which will be the focus of the several complementary methodologies.

Carbohydrate Microarrays will identify candidate ligands and analyze the carbohydrate-binding specificity; **ITC** will evaluate the affinity and thermodynamics of the protein-carbohydrate interaction; **NMR** studies will include protein-ligand interaction studies with the identified carbohydrate ligands using protein chemical shift perturbation and the ligand observed **STD-NMR** technique. **X-Ray Crystallography** methods, more specifically Molecular Replacement or simple difference Fourier, will be used to solve the

3D structure of the protein-carbohydrate complexes and derive the molecular determinants of the interaction.

Besides the hands-on training there will be theory lectures where the main theoretical aspects of each technique will be explained always with an emphasis on the complementarity with the other techniques.

PROGRAM

The seven days course will be composed of theory lectures and practical sessions. These will include hands-on training on various instruments and applications. In the end of each day, an "Integrative" Round Table will be held, where one expert of each methodology discusses practical aspects with students.

Number of participants: 17

Theory Lessons

L1. Carbohydrate microarrays and ligand discovery
L2. ITC and protein-ligand interactions
L3. NMR and protein-ligand interactions
L4-5. NMR and protein-ligand interactions (the ligand viewpoint)
L6-7. NMR and protein-ligand interactions (the protein viewpoint)
L8. X-ray Crystallography and protein-ligand interactions (crystallization, symmetry and spacegroups)
L9. X-ray Crystallography and protein-ligand interactions (X-ray sources, diffraction and data analysis)
L10. X-ray Crystallography and protein-ligand interactions (the phase problem, 3D structure solution, ligand building, refinement and validation)

Hands-on

P1. Training session on Microarray screening analysis and fluorescence imaging
P2. Training session on microarray data analysis and identification of ligands
P3. I. Training session on ITC (sample preparation, starting the experiment) / Planning the experiment; II. Data analysis; III. Cleaning of the apparatus and data analysis
P4. Training session on NMR (sample preparation and acquisition)
P5. Training session on NMR
P6-7. Training session on NMR
P8. Training session on Crystallography (crystallization of protein-ligand complexes)
P9. Training session on Crystallography (X-ray diffraction and data processing)
P10. Training session on Crystallography (Structure solution by MR methods; structure analysis and representation)

PROGRAM SCHEDULE

	Monday - October 6	*Tuesday - October 7*	*Wednesday - October 8*	*Thursday - October 9*
9:00-10:30	Registration and introductory remarks *2.17D* L1: Carbohydrate microarrays and ligand discovery *2.17D*	L2: ITC and protein-ligand interactions *(FMV-UL)*	L3: NMR and protein-ligand interactions *2.17D*	L6: NMR and protein-ligand interactions (the protein viewpoint) *2.17D*
10:30-11:00	*Coffee break (includes poster session, 2nd floor)*			
11:00-13:00	P1: Training session on Microarray screening analysis (Group 1) *4.17* Training session on fluorescence imaging (Group 2) *4.15*	P3: Training session on ITC (sample preparation, starting the experiment) / Planning the experiment *(FMV-UL)*	P4: Training session on NMR (sample preparation and acquisition) *NMR Lab (1st floor)*	P6: Training session on NMR *2.04*
13:00-14:00	*Lunch break (restaurant Campus.come)*			
14:00-15:30	P1 (cont): Training session on Microarray screening analysis (Group 2) *4.17* Training session on fluorescence imaging (Group 1) *4.15*	P3 (cont): Data analysis *(FMV-UL)*	L5: NMR and protein-ligand interactions (the ligand viewpoint) *2.17D*	*Fraser MacMilan*: Invited talk *2.17D*
15:30-16:00	*Coffee break (includes poster session, 2nd floor)*			*Social program (Lisbon tour)*
16:00-18:30	P2: Training session on microarray data analysis and identification of ligands *2.04*	P3 (cont): Training session on ITC (cleaning of the apparatus)/Data analysis (cont) *(FMV-UL)*	P5: Training session on NMR *2.04*	
18:30-19:00	"Integrative" Round Table: one expert of each methodology discusses practical aspects with students *2.17D*			
		Social dinner (Lisbon)		

	Friday - October 10	*Saturday - October 11*	*Sunday - October 12*
9:00-10:30	L8: X-ray Crystallography and protein-ligand interactions (crystallization, symmetry and spacegroups) *2.17D*	L10: X-ray Crystallography and protein-ligand interactions (the phase problem, 3D structure solution, ligand building, refinement and validation) *2.17D*	Selected oral communications (3 participants will be selected for 15-minute oral communications) *2.17D*
10:30-11:00	*Coffee break (includes poster session, 2nd floor)*		
11:00-13:00	P8: Training session on Crystallography (crystallization of protein-ligand complexes) *6.23*	P10: Training session on Crystallography (Structure solution by MR methods; structure analysis and representation) *2.04*	Selected oral communications (3 participants will be selected for 15-minute oral communications) Closing remarks, lunch and departure *2.17D*
13:00-14:00	*Lunch break (restaurant Campus.come)*		
14:00-15:30	L9: X-ray Crystallography and protein-ligand interactions (X-ray sources, diffraction and data analysis) *2.17D*	*João Miguel Dias*: Invited talk *2.17D*	
15:30-16:00	*Coffee break (includes poster session, 2nd floor)*	*Social program (free afternoon)*	

16:00-18:30	P9: Training session on Crystallography (X-ray diffraction and data processing) *1.08 (X-ray Lab) and 2.04*
18:30-19:00	"Integrative" Round Table: one expert of each methodology discusses practical aspects with students *2.17D*

ORGANIZATION AND TEACHING

The course theory and practical lessons are held by members of the different research groups from Associate Laboratory REQUIMTE/UCiBio and the Interdisciplinary Center of Research in Animal Health.

Macromolecular Crystallography

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Glycan Microarrays

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ITC

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Acknowledgments

XTAL-FCT-UNL: Diana Ribeiro, Viviana Correia

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DCC-FCT-UNL: Cristina Groba, Violeta Pereira

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FCT-UNL (http://www.fct.unl.pt)

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CIISA@FMV-UL (http://ciisa.fmv.ulisboa.pt)

PTNMR (http://ptnmr.dq.ua.pt)

PCISBIO (http://xtal.dq.fct.unl.pt/PCISBIO)

INSTRUCT (https://www.structuralbiology.eu)

COST action 1306 - Understanding Movement and Mechanism in Molecular Machines (http://www.cost.eu/domains_actions/cmst/Actions/CM1306)

FCT-MEC (http://www.fct.pt) through financed project RECI/BBB-BEP/0124/2012

ABSTRACTS

Redox control of soluble epoxide hydrolase and heart failure: from structural studies to novel therapeutics

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Human soluble epoxide hydrolase (hsEH) is an enzyme composed of two domains: the N-terminal domain exhibits a phophatase activity that hydrolyses lipid phosphates, whilst the C-terminal domain exerts an epoxide hydrolase activity that converts epoxides, such as epoxieicosatrienoic acids (EETs), to their corresponding diols. EETs are produced by endothelial cells and are able to induce hyperpolarization and therefore relaxation of vascular smooth muscle cells in numerous organs, including the heart. Moreover, EETs have been shown to protect from ischaemic injury and act as anti-inflammatory regulators. Targeting sEH in order to inhibit the conversion of EETs to the inactive dihydroxyeicosatrienoic acids (DHETs) is therefore a promising therapeutic strategy for cardiovascular diseases. A recent study from Eaton lab has demonstrated that electrophilic lipids like 15-Deoxy-Δ-prostaglandin (15d-PGJ_2) and isomers of Nitro-Oleic Acid can stably adduct to sEH, inducing a post-translational modification able to inhibit the enzyme activity. It has been proposed that these molecules bind to the candidate Cys521, since mutant C521S-sEH is insensitive to electrophilic addition reaction. Furthermore, it has been observed that a pre-treatment with hydrogen peroxide can increase the sEH activity as well as the inhibition exerted by the electrophilic lipids. The goal of my project is to understand the mechanism of hsEH inhibition exerted by the electrophilic lipids and to elucidate the exact role of Cys521. Moreover I aim to assess whether the redox state of hsEH is accompanied by conformational changes able to enhance its affinity for substrates and/or inhibitors, thereby influencing its activity and regulation.

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PHOSPHOTRIESTERASE- A PROMISING PROPHYLACTIC FOR OP POISONING?

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The orthophosphate (OP) compounds inhibit acetylcholinesterase (AChE), resulting in over stimulation of synapses. The detoxification of OP compounds can be performed by use of butyrylcholinesterase (BuChE), an enzyme that acts as a scavenger binding OPs, thus reducing their toxic effects. Owing to low Kcat/Km for hydrolysis of OPs, this enzyme needs to be administered in stoichiometric amounts to detoxify OPs. Hence, evolving an enzyme capable of hydrolysing OPs efficiently with catalytic efficiency > 10^7 $M^{-1}min^{-1}$ still remains a major challenge to the protein engineers and crystallographers. Phosphotriesterase (PTE) from *Brevundimonas diminuta* is a promising candidate for OP detoxification. Recently, Cherny *et al*., 2013[1] reported the evolution of PTE to detoxify V agents with > 5,000 fold improvement in comparison to wild type PTE. These improved variants were also tested in guinea pigs[2]. The results of the findings will be presented.

References

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Structural Insights into Terpenoid Biosynthesis

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Terpenoids represent the largest group of natural products on earth. Despite their chemical diversity, the underlying principles of biosynthesis follow strict rules:

A. Polyprenyl diphosphates are cyclised by **class I/II** terpene cyclases forming a hydrocarbon scaffold (1-5 rings, complex stereo chemistry, few heteroatoms)

B. Biological activity is achieved by introducing oxygen atoms via P450 oxidases

C. Subsequently, further chemical moieties are attached by a vast set of different enzymes, e.g. glycosyltransferases

All terpenoids are based on prenyl subunits which are delivered by the universal precursor isopentenyl pyrophosphate (**IPP**) and dimethylallyl pyrophosphate (**DMAPP**). These building blocks are converted into linear polyprenyl diphosphates (C10, C15, C20, C25, C30) which are cyclized by terpene cyclases. Though, the resulting products are extremely diverse (> 60.000 identified compounds), the enzymes catalyzing this reaction are, from a structural point of view, highly conserved. All class I terpene cyclases (the majority of terpene cyclases) activate, as a rule, their substrate by an induced fit mechanism which takes place upon substrate binding (Fig. 1)[1]. After diphosphate abstraction and carbocation formation, the first ring closure takes place, which exclusively forms the anti-Markovnikov product. This is again controlled by the effector- carbonyl group.

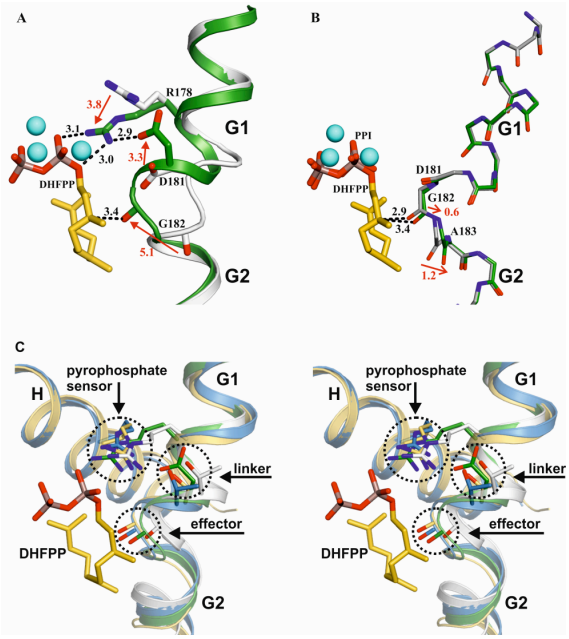

Figure 1 ***Superposition of apo- and ligand bound Selinadiene Synthase reveals induced fit mechanism (A). Alignment of pyrophosphate and dihydro farnesyl pyrophosphate illustrates plasticity of helix G1 (B). Stereoview of different class I terpene cyclases from bacteria, fungi and plants shows the structural conversation of the effector triad (C).***

References

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Mechanistic insights into SOD1 activation by DJ-1 Copper Chaperone

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Both DJ-1 and SOD1 play a protective role against oxidative stress and impaired activity and modified expression of both proteins have been observed in different neurodegenerative diseases. A potential cooperative action of DJ-1 and SOD1 in the same oxidative stress response pathway may be suggested based on a copper-mediated interaction between the two proteins[1,2].
DJ-1 is ubiquitously expressed mainly in the cytosol and functions as a sensor for oxidative stress. SOD1 is a metalloenzyme that catalyzes the disproportionation of superoxide to oxygen and hydrogen peroxide. Its active site requires a copper ion for catalysis while a zinc ion plays a structural role.
To investigate the mechanism underlying the antioxidative function of DJ-1 in relation to SOD1 activity, we studied the ability of DJ-1 to transfer copper ions to SOD1[3]. The copper transfer between the two proteins was investigated through fluorescence spectroscopy, a SOD1 activity assay and through the direct detection of copper bound proteins before and after the metal transfer using electrospray ionization mass spectrometry (ESI-MS). We performed ESI-MS analysis of an incubation mixture of copper-loaded dimeric DJ-1 and monomeric reduced (E,Zn)SOD1 that resulted in the formation of the active dimeric (Cu,Zn)SOD1 and apo DJ-1. Moreover, we observed the formation of an intramolecular disulphide bond between Cys57 and Cys146 in SOD1, which has been reported to be essential for protein dimerization and activity.[4]
On the basis of these preliminary results we aim at investigating the structural modifications that take place during the copper transfer using complementary approaches such as NMR spectroscopy, crystallography and computational simulations. The final goal is to clarify the mechanism through which DJ-1 acts as a copper chaperone in order to unveil potential correlations between its failure and pathological mutations of the two proteins.

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Cyclic peptides to modulate protein-protein interactions

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Vascular endothelial growth factors (VEGFs) belong to the platelet-derived growth factor supergene family, and they play central roles in the regulation of angiogenesis and lymphangiogenesis. VEGF-A, the major factor for angiogenesis, binds to two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), and regulates endothelial cell proliferation, migration, vascular permeability, secretion and other endothelial functions.[1]

VEGF-VEGFR is crucial not only for physiological angiogenesis from early embryonic to adult stages but also for pathological angiogenesis, such as in cancer.

Several anti-VEGF/VEGFR strategies have been developed, including soluble receptors, neutralizing antibodies to VEGF or VEGFRs, other inhibitors of VEGF/VEGFR interaction, such as small molecules inhibiting signal transduction of KDR and antisense oligonucleotides with therapeutic value for a variety of malignancies as well as for other disorders, used alone or in combination with other agents.[2]

In the present work, we present the EXORIS library, synthesized in Prof. Ernest Giralt's lab in order to be tested as possible candidates to modulate different kinds of PPIs. The conformational characterization of these cyclic hexapeptides is described.

The NMR conformational study reveals that the studied peptides have a C2 symmetric conformation. All the studied peptides have the Xaa-D-Pro bonds in the *trans* conformation. Moreover we could conclude that all of them adopt a conformation that contains two β-turns. This was determined by the presence of two hydrogen bonds between the residues preceding the D-Pro residues.

We have also tested these peptides in front of VEGF, in an NMR-based study, and we have found a hit that binds VEGF with mM affinity, which allows us to start a hit to lead optimization process in order to obtain better compounds that bind VEGF with higher affinity.

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Epitope Mapping from STD-NMR: Molecular Recognition of MUC1 Tumour-associated glycopeptides by monoclonal antibodies

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In normal mucin, the extracellular domain is densely covered with highly branched complex carbohydrate structures. In the tumour-associate state, MUC1 can be overexpressed and aberrantly glycosylated, mostly carrying shortened O-glycans,

(ie., Tn, T, Sialyl-Tn or Sialyl-T), as well as a highly immunogenic PDTRP non- glycosylated peptide sequence. [1]

Therefore, MUC1 derivatives have become attractive molecules for the treatment of cancer, because these changes in the glycosylation pattern may represent an excellent starting point for the development of a carbohydrate-based cancer vaccines, educating our immune system to generate antibodies.[2] Therefore, a detailed knowledge of how these glycoconjugates interact with antibodies is of paramount importance to optimize structure-based design of novel types of anticancer vaccines. Indeed, NMR spectroscopy has proved to be notably useful to obtain structural insights into the solution structure and dynamics of ligand-protein complexes.[3] In this context, saturation-transfer difference (STD-NMR), suited to obtain reliable mapping epitopes,[4] was employed to characterize the binding of MUC1-derived (glyco)peptides with distinct monoclonal antibodies (mAbs).

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AROMATIC ALDEHYDES AT THE ACTIVE SITE OF ALDEHYDE OXIDOREDUCTASE FROM *DESULFOVIBRIO GIGAS*

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Molybdenum is a trace element that is crucial for most forms of life. It can be found at the active site of enzymes from both eukaryotic and prokaryotic cells that catalyze key reactions in several important metabolic pathways.[1] *Desulfovibrio gigas* aldehyde oxidoreductase (*Dg*AOR) is a mononuclear molybdenum containing enzyme from the xanthine oxidase (XO) family. It catalyzes the oxidative hydroxylation of aldehydes to the corresponding carboxylic acids.[1] However, its biological role and catalytic mechanism are still poorly understood. In this work[2] steady-state kinetic studies and X-ray crystallography were combined in order to study *Dg*AOR and its interaction with several aromatic aldehydes. X-ray data was obtained for crystals soaked with benzaldehyde, 3-phenyl propionaldehyde and *trans*-cinnamaldehyde. It was possible to identify π-π interactions between the aromatic moiety of benzaldehyde and the side chains of Phe425 and Tyr535 of *Dg*AOR. The crystal structure obtained with 3- phenyl propionaldehyde showed an unspecific reaction of the substrate that binds covalently to His752, blocking the entrance of the substrate channel. This observation explains the suicide-substrate behavior observed by steady-state kinetics under high substrate concentrations. The X-ray data of *Dg*AOR soaked with *trans*-cinnamaldehyde showed a cinnamic acid molecule in the substrate channel and an alternate conformation of the side chain of Phe425, in order to stabilize the product of the reaction.

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DIRECTED EVOLUTION ON FUCO – STRUCTURAL EXPLANATIONS FOR CHANGES IN SUBSTRATE SCOPE

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Propanediol oxidoreductase from *Escherichia coli* (FucO) uses NADH/NAD^+ as cofactors to catalyze the conversion of *S*-lactaldehyde to *S*-1,2-propanediol and vice versa. FucO is an attractive enzyme in the search for possible biocatalysts producing α-hydroxy aldehydes, which are important for the synthesis of natural products and synthetic drugs. Enzymes catalyzing these types of reactions are unique in catalytic power and stereoselectivity. The usage of FucO in synthetic industry is limited by the restricted substrate scope, which makes FucO inactive with larger phenyl-substituted alcohols. We used re-engineering and directed evolution to enable FucO to catalyze the regio- and enantioselective oxidation of arylsubstituted vicinal diols, such as phenylpropanediols, into α-hydroxy aldehyde products[1,2]. We mutated amino acids considered to restrict the entry into the active site, and modeled the mutants that were most active with the substrates phenylacetaldehyde and *S*-3-phenyl-1,2-propanediol and performed docking studies with them. As expected, our experimental and *in silico* results show that the mutations enlarge the active site cavity and enable the mutant enzymes to accommodate the new substrates. We also found specific amino acids in the active site, which need to be conserved to allow the substrates to make stabilizing interactions. Interestingly, an asparagine residue makes the mutant enzymes able to discriminate between phenylacetaldehyde and *S*-3-phenyl-1,2-propanediol. In conclusion, we successfully re-engineered the specialist enzyme FucO to accept also bulkier molecules as substrates, thereby making it more useful for industrial purposes.

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Structural and Functional Characterization of Mab21 Family Members

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Nucleotidyltransferase fold proteins belong to a highly diverse superfamily of proteins and its subfamily Mab21 is constituted by twelve newly assigned members [1]. Most NTases are known to transfer nucleoside monophosphate (NMP) from nucleoside triphosphate (NTP) to an acceptor hydroxyl group belonging to protein, nucleic acid or a small molecule. They share a common core structure, the minimal NTase fold, which has a α/β-fold composed of three-starnded, mixed β-sheet flanked by 4 α-helices. Despite less conserved primary amino acid sequences, residues in the active site are conserved throughout the NTase protein family: hG[GS], [DE]h]DE]h and h[DE]h (h stands for a hydrophobic residue). The best characterized member of the Mab21 family is the cGAMP synthase (cGAS) which upon interaction with DNA synthesizes a cyclic GMP-AMP dinucleotide comprising a 2'-5' and a 3'-5' phosphodiester linkage [2,3]. Production of the second messenger cGAMP by active cGAS leads to production of type I IFN through signaling via STING (stimulator of interferon genes) [4]. cGAS is therefore a pathogen recognition receptor (PRR) that senses cytosolic DNA. Crystal structures of cGAS alone and in complex with DNA, ATP and GTP along with complementary functional studies were carried out in this work in order to explain the broad DNA sensing specificity of cGAS, the DNA-induced structural activation of cGAS and how cGAS catalyses dinucleotide formation [5]. In addition to the characterization of cGAS, our interest expands on the characterization of other Mab21 domain containing proteins.

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Characterization of RNF144A, a RBR E3 Ligase

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The posttranslational modification of proteins by ubiquitin (Ub) is a central regulatory mechanism in many cellular processes ranging from cell cycle regulation to DNA repair[1]. The attachment of Ub to a target protein requires a hierarchical cascade of three enzymes: an Ub-activating enzyme E1, an Ub-conjugating enzyme E2, and an Ub ligase E3. E3 ligases have been classified into three sub-groups, the HECT, RING, and RING-between-RINGs (RBR) type ligases[2]. RBR E3 ligase contains RING1, IBR, and RING2 domains, which transfers Ub from the E2~Ub to the substrate via a RBR~Ub intermediate. RNF144A is a RBR E3 Ligase, targeting cytoplasmic DNA-dependent protein kinase, catalytic subunit for protein ubiquitination and degradation[3]. The RNF144A RBR domain was expressed and purified from *E. coli*. The *in vitro* ubiquitination assay shows that RNF144A can interact with UbcH7 to perform autoubiquitination. The Cys198 in the RING2 domain is the catalytic cysteine residue to form a thioester with Ub. The ubiquitin was chemically conjugated into the functional cysteine residue in UbcH7 by disulphide bond. The ITC assays show that the equilibrium dissociation constants (Kd) of RNF144A:UbcH7 and RNF144A:UbcH7~Ub are 5.95±0.45 and 1.25±0.21 μM, respectively. UbcH7~Ub demonstrates stronger binding with RNF144A than UbcH7. Our work contributes to understanding the mechanism of ubiquitination by RBR E3 Ligases.

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EXPLORING THE ROLE OF SURFACE BINDING SITES IN ONCONASE ANTITUMOR ACTIVITY

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Onconase (ONC), a homologue of ribonuclease A (RNase A), is an 11.8 kDa protein from the oocytes of the Northern leopard frog (*Rana pipiens*). Although ONC and ribonuclease A have 30% amino acid sequence identity and a similar three-dimensional structure[1], the ribonucleolytic activity of ONC is lower than that of RNase A. ONC is at present in phase II/III of clinical trials for the treatment of different types of cancer.

Onconase molecule has ability of binding negatively charged ions. Identified acetate, sulphate and phosphate ion binding sites located on the surface of onconase molecule have important implication for possibly interactions of ONC with anionic membrane components during cancer cell selection and identification, especially with phosphate and sulfate moieties linked to carbohydrates.

Structures of onconase with sulphate (1.15 Å atomic resolution X-ray structure of onconase C30A/C75A variant)[2] and phosphate (unpublished results) ions were determined, but attempts to obtain structural data of a protein-carbohydrate complex were unsuccessful. Thus, the investigation of such interactions between onconase and selected ligands in solution should provide a good basis for future structure based studies of onconase biological function.

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Characterization of the Periplasmic Sulfate-Binding Protein (Sbp) of the phytopathogen *Xanthomonas axonopodis citri*

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Xanthomonas citri is an important phytopathogenic bacterium that causes the canker disease in citrus plants leading to the significant economic losses to Brazil, the second largest exporter of oranges and juice in the world. Understanding the mechanisms of infection and pathogenesis of this bacterium is important to developing ways to control this disease. In recent studies performed by our group, we have identified and functionally characterized proteins belonging to a large family of transporters, which were involved with sulfate, alkanesulfonate, nitrate and taurine transport[1, 2]. Similarly, in *Escherichia coli* these proteins belong to the *cys* regulon that is very well characterized. However, the function and relevance of these proteins in *X. citri* is not clear or even characterized in detail. Specifically, the components of the sulfate uptake and assimilation pathway are interesting for functional and structural analysis since they can be used as targets for development of bacterium growth inhibitors. This work is focused on the expression and purification of the sulfate ABC transporter components from *X. citri* for functional and structural studies. The transporter consists of one periplasmic-binding domain, two transmembrane domains that form a pore for ion passage and two ATPases that generate energy for the transport. Preliminary results based on proteomics and gene reporter analysis, showed the expression of the transporter genes and the activation of the sulfate operon in vitro and in vivo, strongly suggesting this system is important for infection and pathogenesis. The results revealed that Sbp interacts with sulfate with no structural changes but that the interaction induces a significant increasing of the thermal stability. Crystals of Sbp bound to sulfate were obtained and its three-dimensional structure was solved at 3.0 Å resolution with a sulfate bound inside the ligand-binding pocket. ITC preliminary data showed an endothermic reaction whose affinity constant is 7,12 μM.

Altogether these data show the first evidence of the functionality of the ABC transporter for sulfate in *X. citri* and its relevance during infection. In addition, since Sbp is the first protein involved in the ion transport, its structure can be used as a target for the development of inhibitors of the bacterium growth.

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Unveiling the unusual biochemistry of the peroxisomal enzymatic complex involved in ether phospholipids synthesis

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There is increasing evidence that cancer cells show specific alterations in lipid metabolism. These alterations can affect the availability of structural lipids for the synthesis of membranes, the synthesis and degradation of lipids that contribute to energy homeostasis, and the abundance of lipids with signaling functions.
Particularly, the increased level of ether phospholipids is directly involved in certain type of cancer cells (prostate, breast and melanoma) aggressiveness and migration.
Ether phospholipids constitute a special class of phospholipids, presenting an ether bond at the *sn-1* position of the glycerol backbone rather than an ester bond. Although their function is not well understood, it is clear that they play an essential role in membrane dynamics and in cell signaling.
The enzymatic complex involved in the initial committed step in ether phospholipids synthesis is located in peroxisomes and it is composed by dihydroxyacetone phosphate acyl-transferase (DHAP-AT) and alkyl-dihydroxyacetone phosphate synthase (ADPS): the first performs the acylation of DHAP, forming acyl-DHAP, the substrate of ADPS, which in turn exchanges the fatty acid moiety with a fatty alcohol, to form alkyl-DHAP, the precursor of all ether phospholipids.
The activity of these enzymes is so finely tuned, that up-regulation and down-regulation are characteristic of dramatic abnormal conditions, like cancer and *Rhizomelic Chondrodysplasia Punctata*, respectively[1]. Moreover, it has been shown that ether phospholipids level is mainly determined by ADPS and DHAPAT activity, which makes this complex an interesting target for cancer medicine.
Our interest had been, indeed, primarily focused on ADPS, a deeply studied flavoenzyme with a characteristic mechanism of reaction[2], and now it has been extended also to its partner DHAPAT, which has been expressed and purified for the first time, and to their interaction, both from the structural and biochemical point of view.
Having ADPS enzyme in our hands is the starting point for exploring a new area of cancer metabolism and finding new molecules to target ether phospholipids synthesis in cancer cells.

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LSD1-CoREST Demethylase Complex: Biophysical Investigation and Mapping of Nucleosome Recognition Regions

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Human LSD1, Lysine-specific demethylase working on histone tail as chromatin remodeler, is typically found in association with CoREST1 and HDACs, forming a stable module which acts in concert removing the epigenetic marks for gene activation[1]. LSD1 is involved in a large number of physiological processes, from gene silencing to cell-growth and differentiation pathways, as well as many pathological events[2,3]. Despite many biochemical data are available[4], the molecular bases for the substrate recognition by LSD1/CoREST1 (LC) complex are not known.

Indeed, the aim of this current research project is to reveal the molecular details – mostly in terms of protein/protein and protein/DNA – of the assembly and recognition of LSD1/CoREST1 in complex with the nucleosome.

In this regard, the LSD1/CoREST1 heterodimer and its surface mutants have been purified in recombinant form, with the aim of mapping the enzyme-nucleosome interactions. At the same time, fully recombinant nucleosomal particles (NCP) have been purified, and the whole LC-NCP complex reconstituted *in vitro*. The data obtained from SPR and FP led us to develop a starting model for the recognition that involves two steps: the nucleosome is initially recognized by CoREST1 through its SANT2 domain binding to the DNA and this allows the enzyme to recognize the histone tail. Whereas LSD1 active site is able to sense the epigenetic code on H3 tail, CoREST1-DNA binding is strongly affected by electrostatic forces.

While H3 tail and DNA-binding properties of the demethylase complex have been analyzed in depth, one of the main challenges of this work is the stabilization of the complex since it is physiologically transient, especially regarding protein-protein interactions. Some questions still remain unsolved regarding the recognition process. Now we are able to purify the complex and we are directing our efforts to biophysical studies as a base for structural investigations of the whole LC-NCP complex.

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STRUCTURAL AND BIOCHEMICAL CHARACTERIZATION OF ENERGY-COUPLING FACTOR TRANSPORTERS

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In 2006, bioinformatic analysis of prokaryotic genomes resulted in the discovery of a new class of ATP-binding cassette transporters, the energy-coupling factor (ECF-) type ABC transporters.[1] These ECF transporters are found in the membranes of bacteria and archaea, where they mediate the uptake of vitamins, cofactors, amino acids and transition-metal ions. In the last five years, the genomic details as well as the architecture of these membrane proteins have been elucidated.[2-4] Like the classical ABC transporters, the protein complexes consist of two similar nucleotide binding domains, EcfA and EcfA', and two transmembrane domains, EcfT and the S-component, of which the latter functions as substrate binding domain.

Knowledge about the mechanisms of substrate binding and transport is still poor. Crystal structures have been published of substrate bound S-components,[5-7] a nucleotide-free EcfA homodimer[8] and the whole transporter without substrate or nucleotides bound.[3,4] By combining biochemical characterization and x-ray crystallography, we try to identify the individual stages of the transport cycle to elucidate the transport mechanism, using an ECF transporter for folate from *Lactobacillus* as model protein.

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Production and purification of phosphatidylinositol phosphate synthase of *Mycobacterium tuberculosis* for structure determination and search of efficient inhibitors

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Tuberculosis is the second leading cause of death from an infectious disease worldwide.[1] The massive utilization of antibiotics has led to the emergence of strains of *Mycobacterium tuberculosis* resistant to the commonly used therapies.[2] As a result, the development of new antimycobacterial agents is vital. A promising target for drug development is the biosynthetic pathway of phosphatidylinositol, a major phospholipid in *M. tuberculosis* and the lipid anchor of the cell wall components.[3] A key enzyme in this pathway, phosphatidylinositol phosphate synthase (PIPS), is encoded by an essential gene in Mycobateria.[4] Moreover, humans use a distinct biosynthetic pathway that involves a different enzyme, i.e., phosphatidylinositol synthase.[5] Thus, PIPS is a promising drug target. To develop compounds with the ability to inhibit PIPS activity it is essential to know the tridimensional structure of the protein. To this end, a process of producing and purifying PIPS has been established. It is important to note that this is a non-trivial task given the membranar character of the enzyme. First, we constructed a bifunctional enzyme by combining the soluble inositol-1-phosphate cytidylyltransferase (IPCT) with PIPS. This fusion increased the solubility of PIPS and therefore facilitated the purification process. Several expression vectors, *Escherichia coli* strains and growth conditions were tested. The strain leading to maximal expression was grown in LB at 37ºC with 3 hours of induction with 1 mM of IPTG. A yield of 2 mg of pure and active IPCT/PIPS *per* litre of culture was obtained after purification by affinity chromatography. This relatively high yield is an important milestone towards the structural studies and searches for efficient inhibitors. We believe that NMR could be a useful methodology for ligand screening.

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